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14. ABSTRACT

The CAD gene is trifunctional and expresses carbamoylphosphate synthetase/aspartate carbamyltransferase/dihydroorotase, which are required for pyrimidine biosynthesis. TCDD inhibited E2-induced expression of CAD gene and also downregulated E2-mediated transactivation of CAD gene promoter constructs in transient transfection assays. Moreover the CAD gene promoter construct with a mutant DRE was also inhibited by TCDD suggesting that DRE is not important in AhR mediated inhibition of CAD gene expression. Using fluorescence resonance energy transfer (FRET), we show that TCDD inhibits the direct interaction between ERα and Sp1 that is observed in presence of E2. Also at the same time the interaction between ERα and AhR was enhanced in presence of E2 and TCDD in both FRET and co-immunoprecipitation studies. This suggests that AhR may act as a corepressor and thereby inhibits ERα/Sp1 mediated responses in breast cancer cells. Also E2-induced CAD gene expression and CAD promoter constructs was down regulated by PPAR gamma agonist suggesting a possible cross talk between ER and PPAR gamma signaling pathways.

15. SUBJECT TERMS

Breast Cancer

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Introduction

The hormone 17β-estradiol (E2) and related estrogenic hormones play an important role in several physiological processes, including development of the female and male reproductive tracts as well as bone, vascular, and neuronal function. The environmental toxicant TCDD mediates its responses through the aryl hydrocarbon receptor (AhR) and inhibits a broad spectrum of estrogen-induced responses in the uterus and in human breast cancer cells The aim of this study was to understand the mechanism of CAD gene expression and to investigate the mechanisms underlying ER-AhR crosstalk and also interaction between PPAR gamma and ER signaling pathways using the CAD gene as the model. Previous work in this laboratory showed that the trifunctional CAD gene is induced by E2 in MCF-7 or ZR-75 breast cancer cells and hormonal regulation of CAD gene is regulated by a nonclassical DNA-independent mechanism that involves ERα-Sp1 (protein-protein) interactions at E2-responsive GC-rich promoter motifs. In this study, our results showed that TCDD inhibited E2-induced CAD gene expression and also E2-mediated transactivation of CAD gene promoter constructs. TCDD inhibited E2-induced luciferase activity in ZR75 cells transiently transfected with pCADm3, a construct of CAD gene promoter containing mutations in a potential inhibitory DRE, suggesting that this element was not responsible for TCDD mediated inhibition. The proteasome inhibitor MG132 alone significantly blocked basal and E2-induced CAD mRNA levels and it was not possible to directly determine the effects of ERα restoration on AhR-mediated downregulation of ERα. Analysis by fluorescence resonance energy transfer (FRET) showed that interaction between ERα and Sp1 in the presence of E2 was inhibited after cotreatment with TCDD. Also analysis by FRET and co-immunoprecipition studies showed that the direct interaction between ER and AhR was enhanced in presence of E2 plus TCDD. These results suggest a possible role for the AhR as a corepressor of ERα/Sp1-mediated activation of the CAD gene promoter. The AhR modulates multiple pathways and is a target for development of selective AhR modulators (SAhRMs) for treatment of breast cancer. E2-induced CAD gene expression and CAD promoter constructs was also downregulated by PPAR gamma agonist suggesting possible crosstalk between ER and PPAR gamma signaling. Results of this study will increase our understanding of the complex processes underlying the diverse actions of E2 and facilitate strategies for development of mechanism-based drugs for treatment of breast cancer.

BODY

This project has been focused on the inhibitory effects of AhR agonists and peroxisome proliferator activated receptor gamma (PPAR γ) agonists on E2- induced expression of carbamoyl phosphate synthetase, aspartate transcarbamoylase and dihydroorotase (CAD) in breast cancer cells. Based on the results of preliminary studies we investigated the inhibitory effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on CAD gene expression (as described in Task 2). We also studied the effects of PPAR gamma agonists such as PGJ2, Rosiglitazone and a new class of PPAR gamma agonists 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methanes on E2-induced CAD gene expression as described in task 1 of statement of work.

Previous results showed that the hormone-responsive region of the CAD gene promoter was primarily associated with the upstream –90/+25 sequence which contains 3 GC-rich elements that bind Sp proteins (Fig. 1)

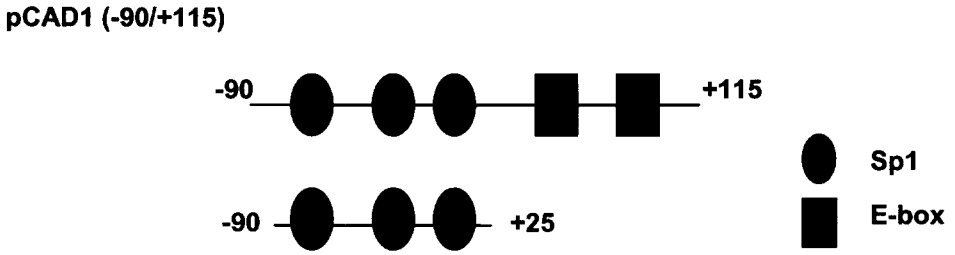


Figure 1. pCAD1 has 3 GC-rich elements and two E-boxes. Hormone responsiveness was found to be associated with two upstream GC-rich sites.

Our results indicated that E2 induces CAD gene expression in ZR75 and MCF7 breast cancer cells and this response is mediated through interaction of ERα/Sp1 with proximal GC-rich motifs (Khan *et al*).

Inhibition of hormone-induced CAD gene expression by ICI 182780 and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Task 2)

The effects of TCDD and ICI on CAD mRNA levels were determined in ZR75 cells (Fig 2A). ZR75 cells were treated with 10 nM E2 for 1, 3, 6, 12, and 24 h or cotreated with TCDD plus E2, E2 plus ICI, TCDD and ICI for 12 h and CAD mRNA levels were determined at all time points. Results in Figure 2A show that E2 induces CAD mRNA levels in ZR75 cells and this response is inhibited by the anti-estrogen ICI 182,780 and the Ah receptor agonist TCDD. Figure 2B compares the effects of E2, TCDD and E2 plus TCDD treatment on CAD gene expression in ZR75 cells pretreated with DMSO (solvent control) or the proteasome inhibitor MG132. Previous reports showed that the anti-estrogenic activity of TCDD on some genes was due to limiting levels of ERα in cells cotreated with E2 plus TCDD due to activation of proteasome-dependent degradation of ERα (Wormke *et al*). The results in Fig 2B indicate that the proteasome inhibitor alone

significantly blocked basal and E2-induced CAD mRNA levels and it was not possible to directly determine the effects of ERα restoration on reversal of Ah-responsiveness. The results do not exclude a role for ERα depletion in mediating the inhibitory effects of TCDD on induction of CAD mRNA by E2.

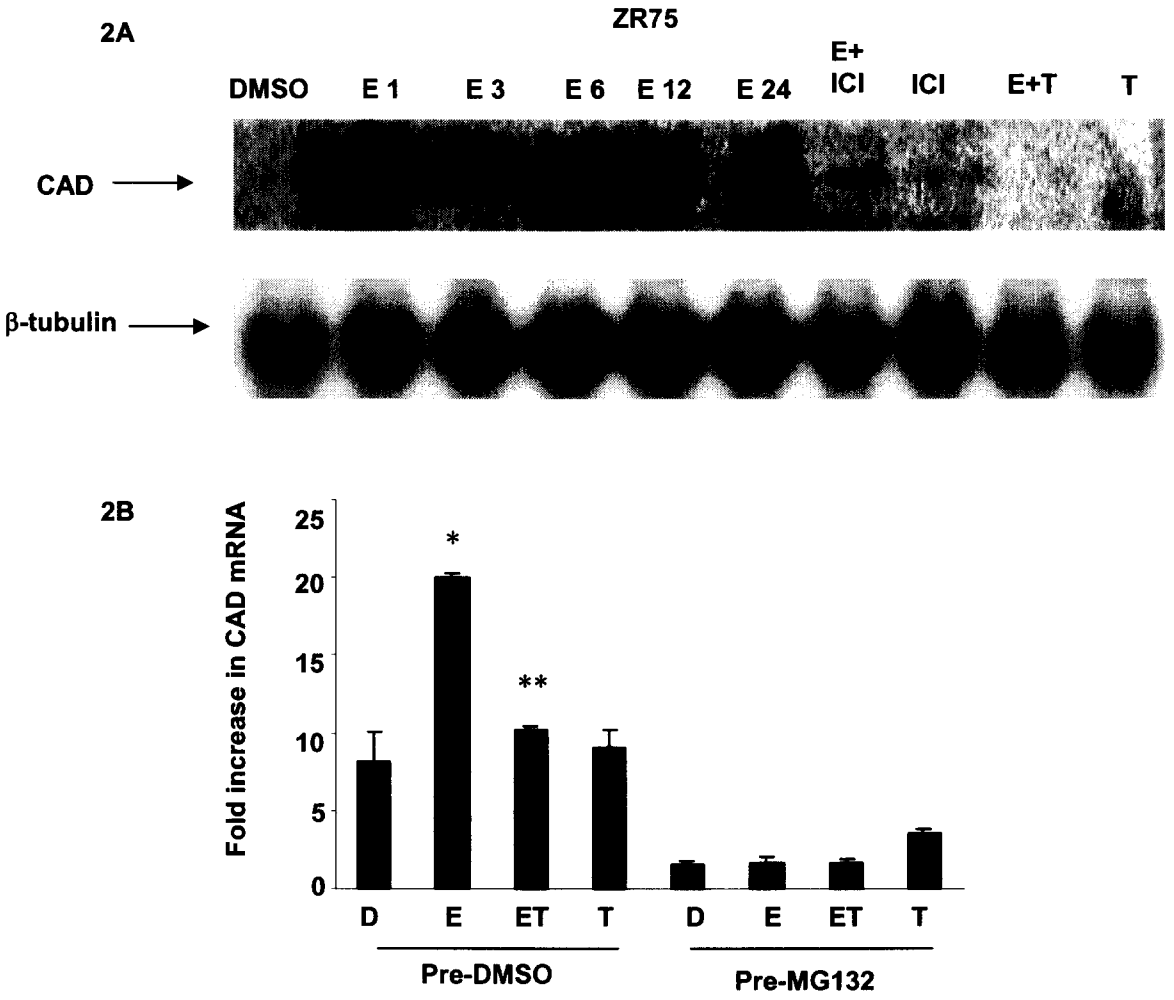
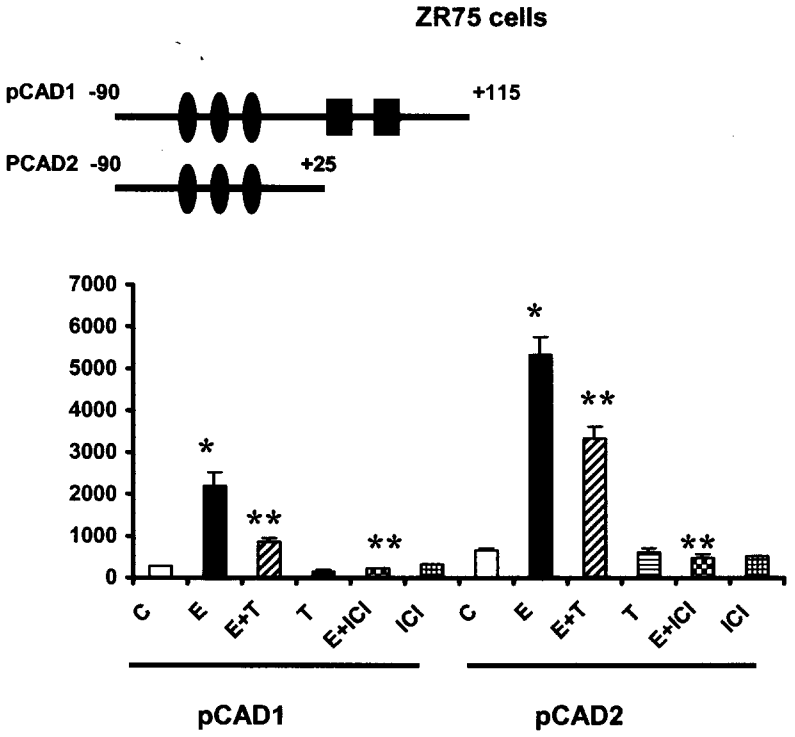


Figure 2. A. Northern analysis of CAD mRNA from ZR-75 cells. The cells were treated with DMSO for 24 h (lane 1) and 10 nM E2 for 1, 3, 6, 12, and 24 h (lanes 2–5, respectively) or co-treated with E2 and ICI (lane 7) or E2 and TCDD (lane 9) or ICI (lane 8) and TCDD (lane 10) alone for 12 h. Cell extracts were obtained, and total RNA was isolated and subjected to Northern analysis. The intensity values were normalized to the values of β-tubulin mRNA. B. Effects of proteasome inhibitor MG132 on TCDD down-regulation of CAD gene expression in ZR75 cells. ZR75 cells were pretreated with DMSO or 10 μM MG132 for 30 min before treating with DMSO, 10 nM E2, E2+TCDD or 10 nM TCDD for 12 h. Cell extracts were obtained, and total RNA was isolated and subjected to Real time PCR analysis using SYBR green. The fold induction of CAD mRNA levels was calculated and normalized to TBP mRNA levels. Significant induction is shown by asterisk (*) and downregulation is represented as (**).

Inhibition of hormone-induced transactivation of CAD gene promoter reporter constructs by ICI 182780 and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

The inhibitory effect of TCDD on E2-induced transactivation was investigated in ZR75 (Fig. 3A) and MCF-7 (Fig. 3B) cells transfected with constructs containing CAD gene promoter inserts. E2 induced transactivation in cells transfected with pCAD1 (-90/+115) and pCAD2 (-90/+25) and after cotreatment with E2 plus TCDD or ICI 182780, the induced response (luciferase activity) was significantly decreased.

3A



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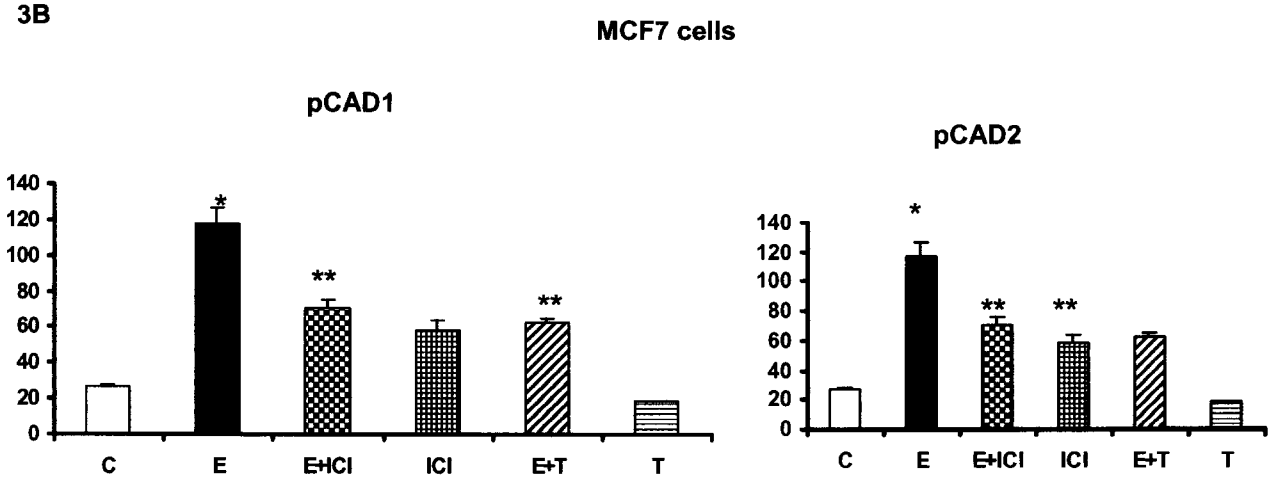
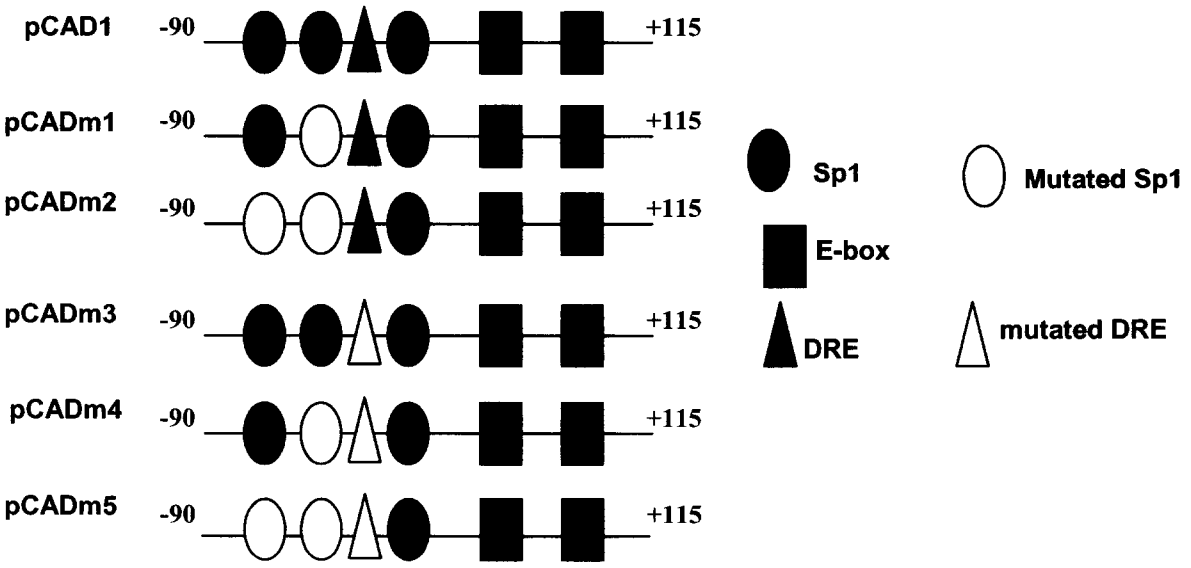


Figure 3. Inhibition of estrogen induced transactivation of CAD gene promoter constructs by TCDD and ICI in ZR75 (A) and MCF7 (B) cells. ZR75 or MCF7 cells were transfected with pCAD1 or pCAD2 constructs, dosed with DMSO (C), 10 nM E2, 10 nM TCDD, 1 μM ICI or combined treatments and luciferase activities were determined. Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction is indicated with an *asterisk* and downregulation is represented as (**).

The upstream GC-rich sites were previously identified as the major E2-responsive motifs in the CAD gene promoter and the results in Figure 4B demonstrate that E2 did not induce transactivation in ZR75 cells transfected with pCADm1 or pCADm2 and TCDD +/_ E2 also did not affect transactivation. Inhibitory AhR-ERα crosstalk for cathepsin D, heat shock protein 27 and c-fos has been linked to direct interactions of the AhR complex with inhibitory DREs (iDREs) containing the core CACGC motif that binds the AhR complex. The CAD promoter also contains a CACGC motif however E2 induced luciferase in ZR75 cells transfected with pCADm3 (mutated DRE) and in cells cotreated with E2 plus TCDD the induced response was significantly inhibited. Transfection of plasmids containing mutations of GC-rich sites (pCADm4 or pCADm5) resulted in loss of E2-responsiveness and were not affected by TCDD. These results suggest that the indirect antiestrogenic activity of TCDD was iDRE independent.

4A



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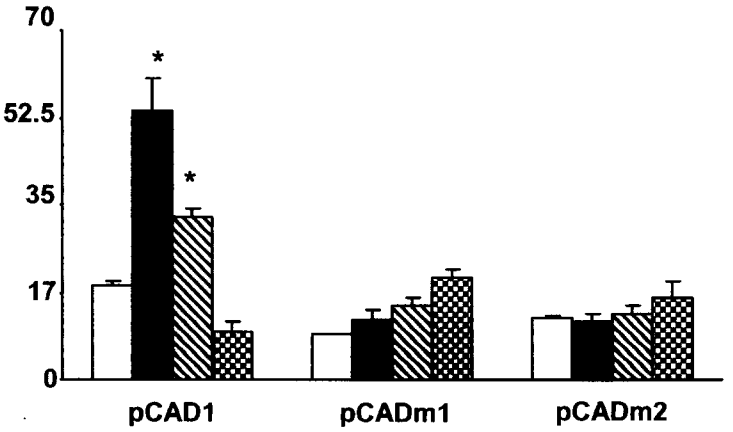


4C

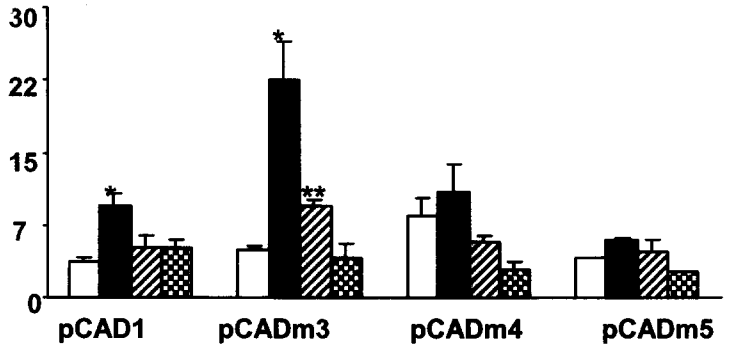
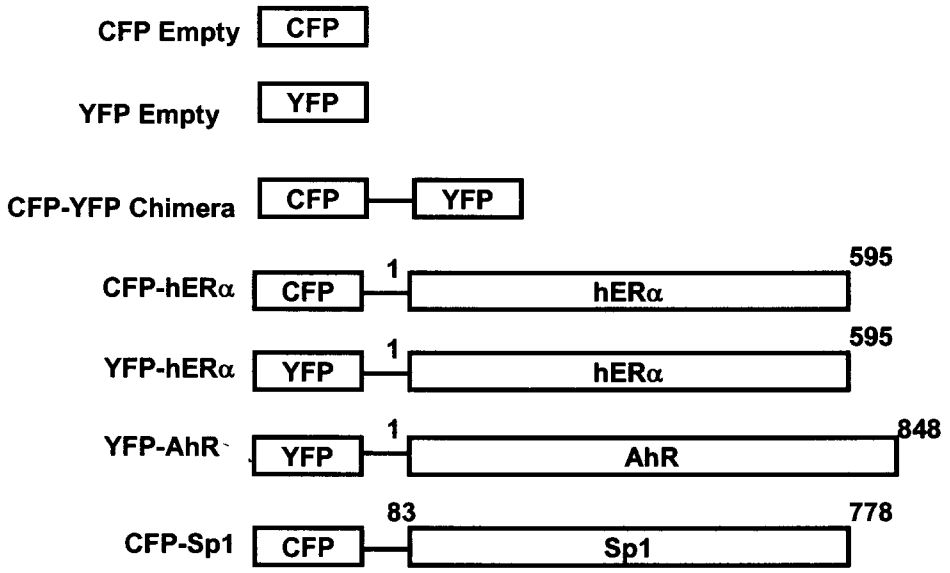


Figure 4. A. CAD gene promoter mutant constructs. B. Inhibition of estrogen induced transactivation of CAD gene promoter constructs by TCDD in ZR75 cells (B & C). ZR75 cells were transfected with CAD gene promoter constructs, dosed with DMSO (D), 10 nM E2, 10 nM TCDD, or E2+TCDD and luciferase activities were determined. Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction is indicated with an *asterisk.*

Protein-protein interactions between AhR and ERα and also ERα and Sp1 in living cells in presence of ligands using flourescence resonance energy transfer (FRET)

In order to further understand the AhR/ER crosstalk we used FRET to study protein protein interactions in presence of ligands. FRET has been used to study interactions of nuclear receptors and coregulators or peptides in living cells. Using CFP/YFP chimeras of ERα and Sp1, we observed ligand-induced interactions of ERα and Sp1 in MCF7 cells (Kim *et al*). Fig 5A summarizes the YFP/CFP chimeras used in this study in MCF7 cells. Figure 5B summarizes the effects of DMSO, E2, E2 plus TCDD or TCDD on distribution of transfected YFP-AhR in MCF7 cells which are ERα and AhR positive and COS-1 cells that do not express ERα or AhR. In both MCF7 and COS-1 cells, treated with DMSO & E2 the AhR was detected in the cytosolic and nuclear fractions whereas after treatment with E2 plus TCDD or TCDD alone the AhR was localized exclusively in the nucleus and exhibited a punctate staining pattern. In a previous report, the YFP/CFP ER and Sp1 chimeras were functional in transactivation assays (kim et al). The functionality of the YFP-AhR was also investigated in COS-1 cells (figure 5C) treated with DMSO or 10 nM TCDD and transfected with pDRE which contains three tandem consensus DREs linked to firefly luciferase. In the absence of YFP-AhR TCDD did not induce luciferase activity however induction by TCDD was observed after cotransfection of YFP-AhR indicating that the chimeric AhR-YFP was functional.

5A


CFP Empty
CFP
YFP Empty
YFP
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CFP
YFP
CFP-hERα
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595
CFP
hERα
YFP-hERα
1
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848
YFP
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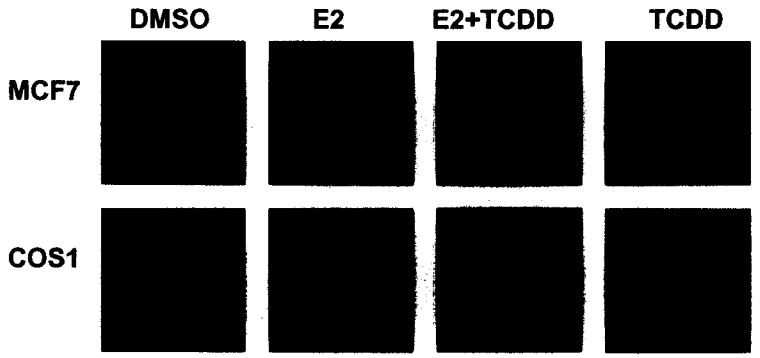
CFP-Sp1
83
778
CFP
Sp1

5B


DMSO
E2
E2+TCDD
TCDD
MCF7
COS1

5C

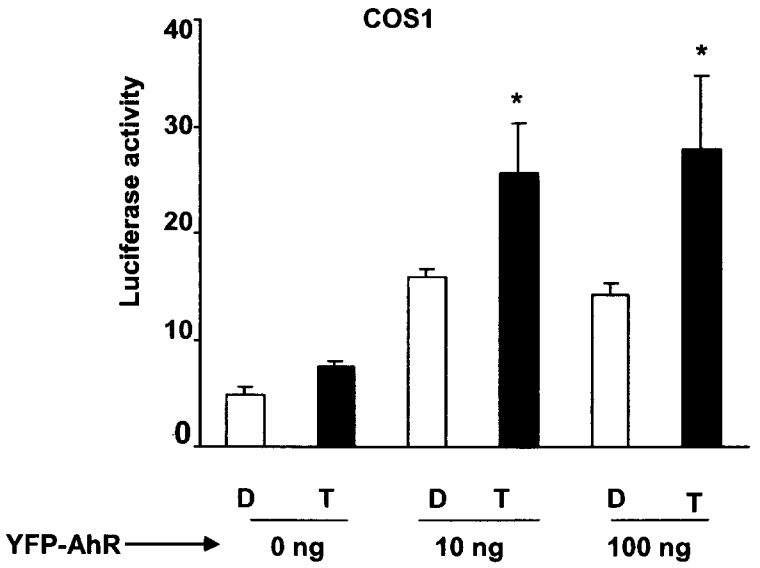
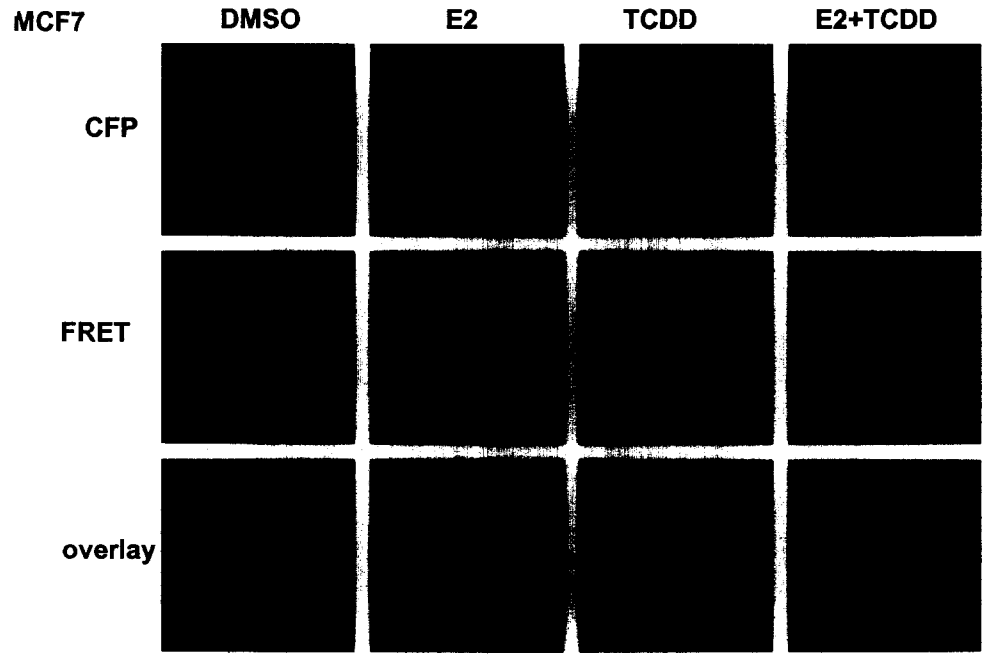

COS1
Luciferase activity
40
30
20
10
0
*
*
D
T
D
T
D
T
YFP-AhR
0 ng
10 ng
100 ng

Fig. 5. YFP-AhR functionality in MCF7 and COS1 cells. A. MCF7 and COS1 cells were transfected with 250 ng of YFP-AhR for 16 h and then dosed with different treatments for 30 min before acquiring images. B. Induction of DRE-luc in COS1 cells. COS1 cells were co-transfected with DRE-luciferase construct, 50 ng βgal along with different amounts of YFP-AhR. Cells were then treated with DMSO (D) or TCDD (T) for 24 h, luciferase activities in the various treatment groups were performed on 30 µl of cell extract using the luciferase assay system, and results were normalized to β-galactosidase enzyme activity. Significant ($p < 0.05$) induction by E2 is indicated with an asterisk.

Ligand-induced interactions between YFP-AhR and CFP-ERα in living cells using FRET

The direct interactions of CFP-ER and YFP-AhR were determined by FRET in MCF7 (figure 6) and COS1 (figure 7) cells. Excitation of CFP-ER at 410 nm and emission at 488 nm illustrates the blue fluorescent emission of nuclear CFP-ER. The yellow fluorescence was detected in the FRET channel at 525 nm and this represents CFP-YFP interaction and energy transfer. The energy transfer and FRET will take place only if the two proteins are within the distance of (1-10 nm). Figure 6B and 7B quantitate the FRET efficiencies in the various treatment groups. There was a significant increase in FRET efficiencies in cells treated with E2, TCDD and TCDD plus E2. The overlay of the CFP and FRET signals are shown in Figures 7A and 8A and confirm the enhanced emission in the treatment groups. The results demonstrate for the first time that AhR and ERα interact in living cells and this observation is consistent with in vitro studies that also show interactions between these proteins.

6A



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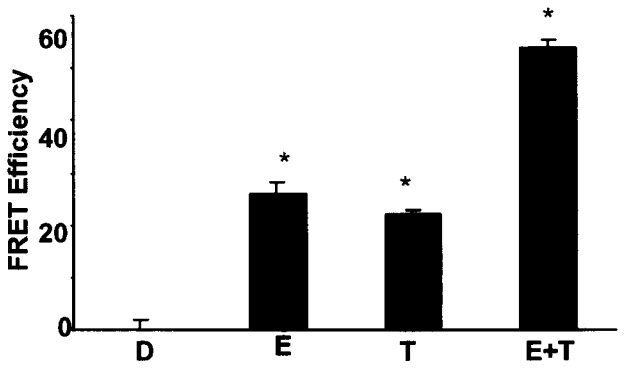
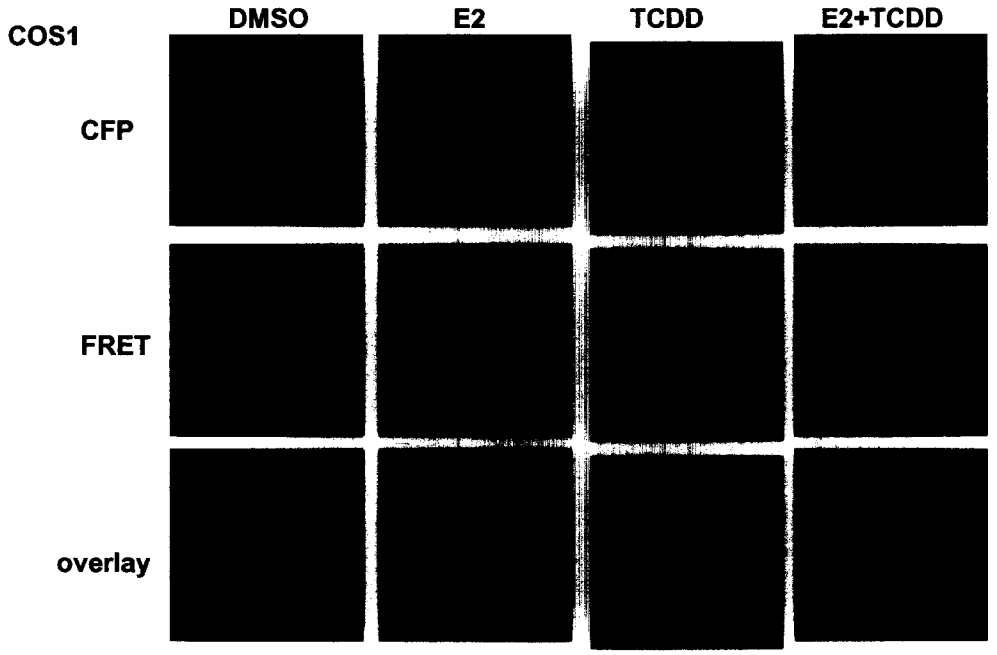


Fig. 6. Analysis of ERα/AhR interaction in MCF7 cells using FRET. A. FRET images from cells transfected with CFP-ER and YFP-AhR. MCF7 cells were co-transfected with CFP-ER and YFP-AhR for 16 h; to acquire images, cells were pretreated with TCDD for 10 min before treating with DMSO or E2 for 8 min. Images were then acquired from 8 min to 18 min. B. FRET efficiency of CFP-ER /YFP-AhR. Ten to fifteen images were acquired per treatment, and each image contained one to five cells to be analyzed. The subtraction of background signal from the images was carried using CFP-YFP chimera as a positive control. Significant ($P < 0.05$) induction compared with DMSO is indicated as (*)

7A



7B

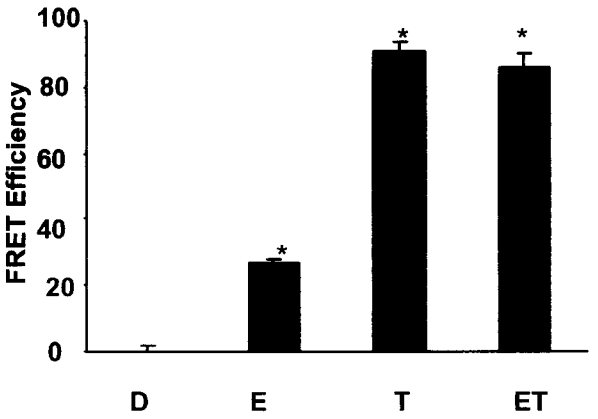
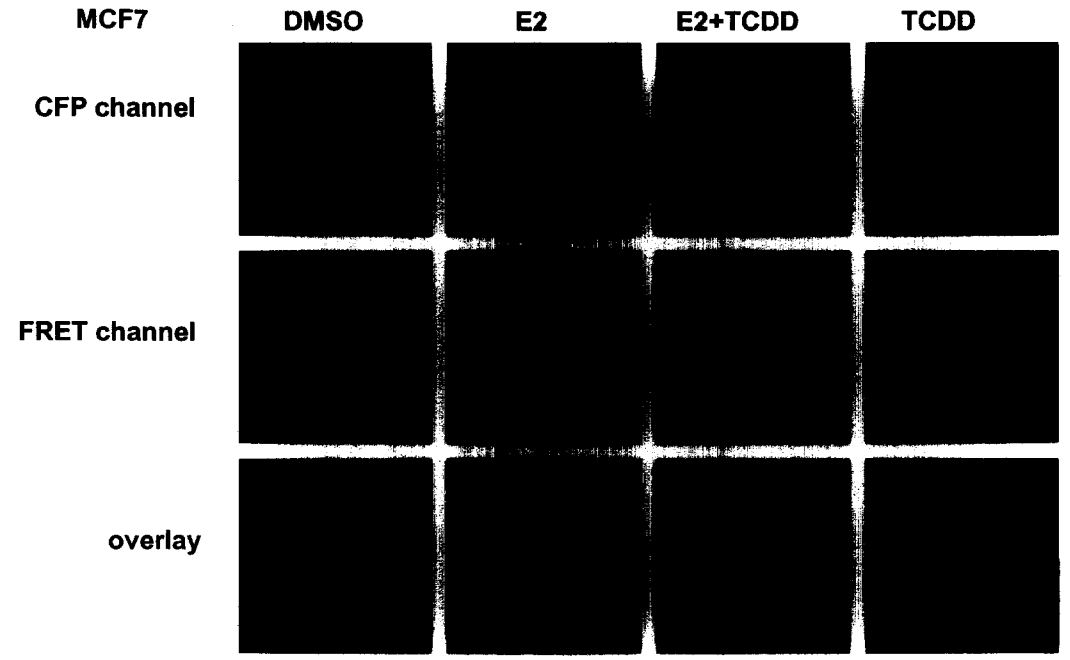


Fig. 7. Analysis of ERα/AhR interaction in COS1 cells using FRET. A. FRET images from cells transfected with CFP-ER and YFP-AhR COS1cells were co-transfected with CFP-ER and YFP-AhR for 16 h; to acquire images, cells were pretreated with TCDD for 10 min before treating with DMSO or E2 for 8 min. Images were then acquired from 8 min to 18 min. B. FRET efficiency of CFP-ER /YFP-AhR. Ten to fifteen images were acquired per treatment, and each image contained one to five cells to be analyzed. The subtraction of background signal from the images was carried using CFP-YFP chimera as a positive control. Significant ($P < 0.05$) induction compared with DMSO is indicated as (*)

Protein-protein interactions between YFP-ERα and CFP-Sp1 in living cells in presence of ligands using FRET

Direct interactions between chimeric AhR and Sp1 proteins were not observed in the FRET assay due to the high molecular weights of these proteins therefore we investigated the effects of the liganded AhR complex on hormone-dependant activation of ERα/Sp1 in MCF-7 cells which express endogenous AhR. Cells were transfected with CFP-Sp1 and YFP-ERα and treated with solvent (DMSO) control, E2, TCDD or TCDD plus E2; Cells were pretreated with TCDD for 10 minute prior to addition of E2 for 8 min (Fig. 8A). Cells treated with DMSO or TCDD exhibit low FRET efficiencies whereas after treatment with E2 there was a significant increase in the FRET signal. This ligand-dependant increase was consistent with our recent FRET study showing ERα-Sp1 interactions in breast cancer cells (Kim *et al*). However, the intensity of the E2 induced FRET emission is significantly decreased in cells treated with E2 plus TCDD and quantitation of the FRET efficiencies summarized in Figure 8B confirms this observation. These results demonstrate that the ligand AhR complex induces a rapid change in ERα/Sp1 interactions on the CAD gene/gene promoter (Figs. 2-5)

8A



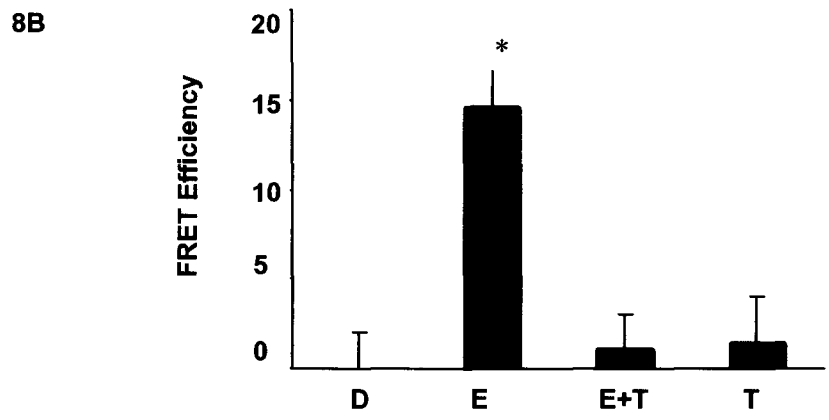


Fig. 8. Analysis of ERα/Sp1 interaction in MCF7 cells using FRET. A. FRET images from cells transfected with CFP-Sp1 and YFP-ER. MCF7 cells were transfected with CFP-Sp1 and YFP-ER for 16 h; to acquire images, cells were pretreated with TCDD for 10 min before treating with DMSO or E2 for 8 min. Images were then acquired from 8 min to 18 min. B. FRET efficiency of CFP-Sp1 /YFP-hER. Ten to fifteen images were acquired per treatment, and each image contained one to five cells that were analyzed. The subtraction of background signals from the images was carried using CFP-YFP chimera as a positive control. Significant ($P < 0.05$) induction compared with DMSO is indicated as (*).

Co-immunoprecipiation to study protein interactions

We further investigated ER-AhR interactions treated with DMSO, E2, TCDD and TCDD plus E2 and in MCF-7 cells transfected with FLAG-AhR. Cell lysates were immunoprecipitated with non-specific IgG or FLAG antibodies and analyzed for ERα by Western blot analysis. ERα was detected in IgG precipitates and the low levels were observed in the E2 plus TCDD treatment group. Interactions of ERα with the AhR were determined in the FLAG antibody immunoprecipitated in which higher levels of ERα were observed in the TCDD and TCDD plus E2 treatment groups and these results were consistent with the enhanced AhR-ERα interactions observed by FRET in cells treated with E2 and E2 plus TCDD (Fig. 9).

These results suggest that the AhR complex either forms a transcriptionally-inactive AhR:ER/SP1 complex where the AhR corepresses ER/SP1 or the AhR competitively dissociates ERα from SP1. The latter pathway is supported, in part, by recent studies showing that TCDD/TCDD+E2 recruits the ER/AhR complex to promoters of Ah responsive genes such as CYP1A1.

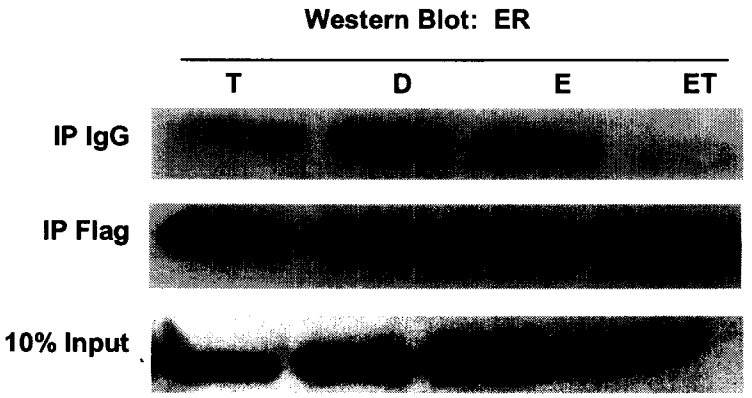


Fig. 9. Interaction between ERα and AhR-FLAG protein is enhanced in presence of E2+TCDD. MCF7 cells were transfected with hAhR-FLAG and pcDNA3-ERα for 18 h and treated with D, E, ET or T for 30 min. Nuclear extracts were isolated and immunoprecipitated with anti-FLAG antibody or mouse IgG antibody. ERα protein was then analyzed by Western blot (WB) analysis.

Studies in progress for Task 2:

- Extensive in vivo chromatin immunoprecipitation (CHIP) assays to determine and confirm the binding of proteins to CAD gene promoter in response to E2, TCDD and E2 plus TCDD are in progress.
- To determine which Sp protein is involved in E2 induction and TCDD down regulation, studies using small inhibitory RNA for Sp1, Sp3 and Sp4 in transient transfection assays will be carried out to complete Task 2 of the project.

PPAR gamma–dependent inhibition of E2-induced CAD gene expression

The effect of PPAR gamma agonist PGJ2 on CAD mRNA levels were determined in ZR75 cells (Fig. 10). ZR75 cells were treated with 10 nM E2, 10 μM PGJ2, 20 μM PGJ2 or E2+PGJ2 for 12 h and mRNA levels were determined. Significant induction by E2 was observed after 12 h and this was significantly down regulated by PGJ2.

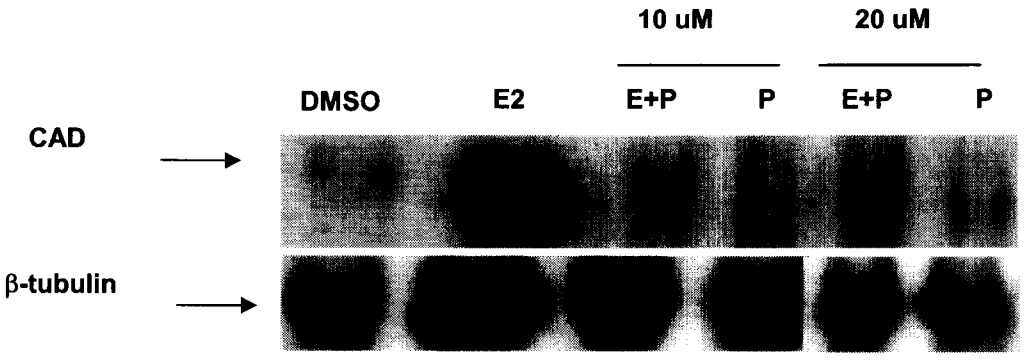
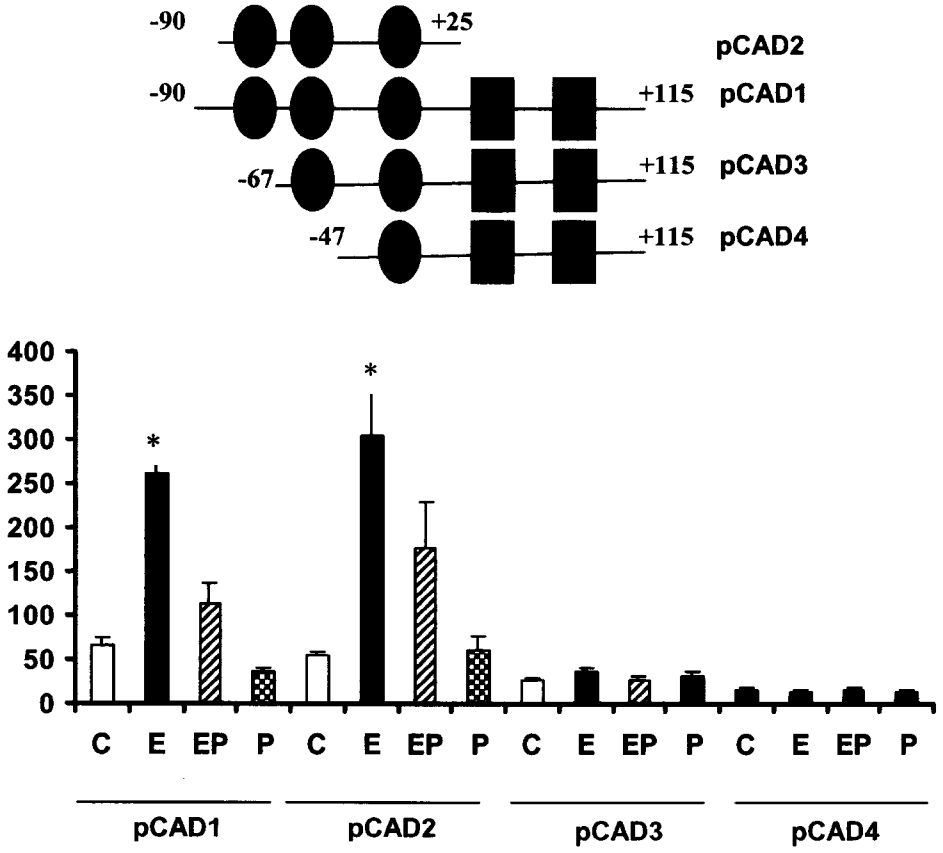


Figure 10. Effect of PPAR gamma agonist PGJ2 on E2-induced CAD gene expression in ZR75 cells. The cells were treated DMSO, 10 nM E2, 10 μM PGJ2 (P) or E2+PGJ2 for 12 h. Cell extracts were obtained, and total RNA was isolated and subjected to Northern analysis. The intensity values were normalized to the values of β-tubulin mRNA.

Inhibition of hormone-induced transactivation of CAD gene promoter reporter constructs by PPAR gamma agonist PGJ2

The inhibitory effect of PGJ2 on E2-induced transactivation was investigated in MCF-7 (Fig. 11) cells transfected with constructs containing CAD gene promoter inserts. E2 induced transactivation in cells transfected with pCAD1 (-90/+115) and pCAD2 (-90/+25) constructs and after cotreatment with E2 plus PGJ2, the induced response (luciferase activity) was significantly decreased. Constructs containing deletion of GC-rich sites [pCAD3 (-67/+115) and pCAD4 (-47/+115)] were not induced by E2. Also the constructs containing point mutations in the functional GC-rich site in pCADm1 was downregulated by PGJ2 whereas pCADm2 which contains point mutations in both upstream GC-rich sites was not affected by E2 or PGJ2. These results suggest a possible crosstalk between ER and PPAR gamma signaling pathways and research is in progress to further characterize these effects.



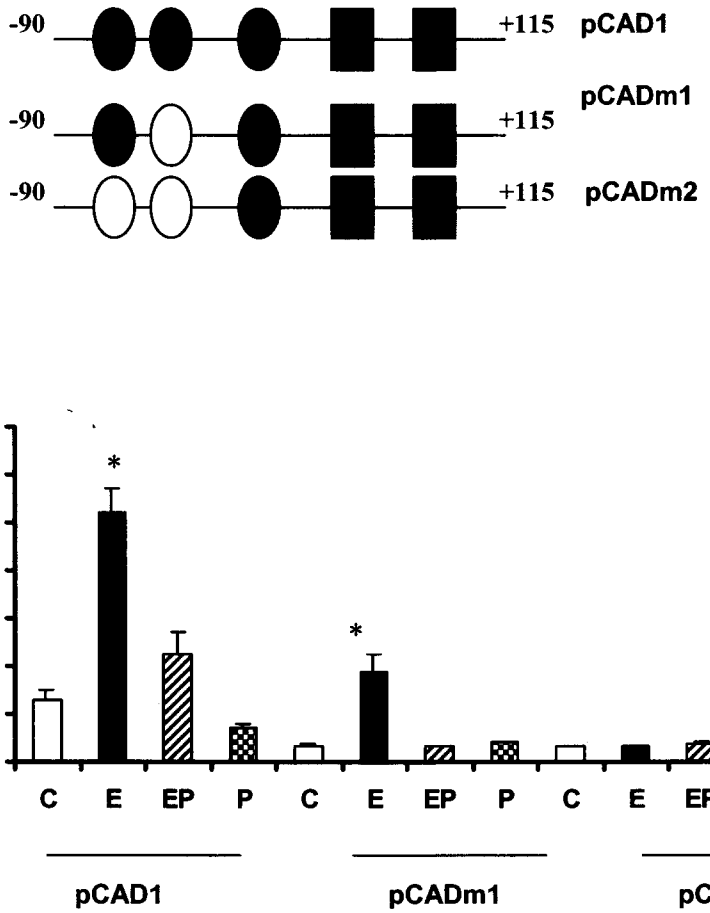


Figure 11. Inhibition of estrogen induced transactivation of CAD gene promoter by PGJ2 in MCF7 cells. MCF7 cells were transfected with CAD promoter constructs, dosed with DMSO (C), 10 nM E2, 10 μM PGJ2 plus E2 or PGJ2 alone and luciferase activities were determined. Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction is indicated with an *asterisk.*

Studies in progress for Task 1

- Perform Real time PCR to determine the time-dependent effects of 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methanes on CAD mRNA levels in breast cancer cells.
- Extensive in vivo chromatin immunoprecipitation (CHIP) assays to determine and confirm the binding of proteins to CAD gene promoter in response to PPAR gamma agonists.
- To determine which Sp protein is involved in down regulation using small inhibitory RNA for Sp1, Sp3 and Sp4 in transient transfection assays.

Key Research accomplishments

- Significant induction of CAD gene expression by E2 was observed after 12 and 24 h and this induction was significantly downregulated by both TCDD and the pure anti-estrogen ICI

- The roteasome inhibitor MG132 alone significantly blocked basal and E2-induced CAD mRNA levels and it was not possible to directly determine the effects of ERα restoration on AhR-mediated effects.

- TCDD significantly decreased E2-induced transactivation of CAD gene promoter constructs with GC-rich elements in ZR75 cells. TCDD also inhibited E2-induced luciferase activity of pCADm3, containing mutations in a potential DRE, suggesting that this element alone was not responsible for TCDD mediated inhibitory responses.

- Analysis by FRET showed that the direct interaction observed between ERα and Sp1 in the presence of E2 was inhibited after cotreatment with TCDD. Also Analysis by FRET and co-immunoprecipition studies showed that direct interactions between ERα and AhR was enhanced in presence of E2 plus TCDD

- These results suggest a possible role for the AhR as a corepressor for ER/Sp1-mediated activation of the CAD gene promoter.

- The PPAR gamma agonist PGJ2 significantly downregulated E2-induced CAD gene mRNA levels and transactivation of CAD gene promoter constructs suggesting a possible cross talk between PPAR gamma and ER signaling pathways.

Reportable Outcomes

(a) Manuscripts, abstracts, presentations

Khan, S., Abdelrahim, M., Samudio, I. & Safe, S. Estrogen receptor/Sp1 complexes are required for induction of cad gene expression by 17beta-estradiol in breast cancer cells. *Endocrinology.* 144, 2325-2335 (2003).

Poster Presentation at American Association for Cancer Research (2005):
Crosstalk: Aryl Hydrocarbon Receptor And Estrogen Receptor signaling Pathways In Breast Cancer Cells using CAD Gene As the Model.
S. Khan, K. Kim, R. Barhoumi, and S. Safe.

(b) Patents/licences applied for or issued
None.

(c) Degrees
Ph.D in progress (Shaheen Khan)

(d) Cell lines/serum
No new lines developed.

(e) Informatics
None.

(f) Funding applied
None.

(g) Employment/research opportunities
None.

Conclusions

Results of this study have demonstrated that estrogen-induced CAD gene expression was downregulated by AhR agonist TCDD. The intensity of the E2 induced FRET emission between ERα and Sp1 was significantly decreased in cells treated with E2 plus TCDD demonstrating that the ligand AhR complex induces a rapid change in ERα/Sp1 interactions on the CAD gene/gene promoter. Moreover enhanced AhR-ERα interactions observed by co-immunoprecipitation and FRET in living cells treated with E2 and E2 plus TCDD suggest that the AhR complex either forms a transcriptionally-inactive AhR:ER/SP1 complex where the AhR corepresses ER/Sp1 or the AhR competitively dissociates ERα from SP1. Moreover PPAR gamma agonist PGJ2 also decreased E2-induced CAD gene expression suggesting ER and PPAR gamma crosstalk. Results of this study will increase our understanding of the complex processes underlying the diverse actions of E2 and facilitate strategies for development of mechanism-based drugs for treatment of breast cancer.

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